The Hidden side of SERPINB1/Leukocyte Elastase Inhibitor

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Serpins: inhibitors of proteases

SERPINB1, also called Leukocyte Elastase Inhibitor (LEI) is a member of the clade B of the serpin (serine protease inhibitors) superfamily of proteins.[1].

Serpins are the largest and most broadly distributed superfamily of protease inhibitors. All multicellular eukaryotes have serpins: humans, Caenorhabditis elegans, Drosophila, Arabidopsis thaliana. They count about 36, 9, 13 and 29, serpin genes, respectively [2]. In contrast, most prokaryotes have only a single gene. The serpin superfamily has been divided in higher animals into 9 clades (A-I). All the clades contain extracellular proteins, except for clade B, which contains predominantly intracellular proteins [3].

The majority of serpins inhibit serine proteases, but serpins that inhibit caspases or papain-like cysteine proteases have also been identified [4-5]. Inhibitory serpins are 'suicide' or 'single use' inhibitors that use a unique conformational change to inhibit proteases. This conformational transition is termed the 'stressed (S) to relaxed (R) transition'. Native serpins are in fact trapped in an metastable state, rather than their most stable conformation (they represent an exception to Anfinsen's law) [6].

Other functions of serpins

Sometimes serpins perform a non-inhibitory function. Several human serpins function as molecular chaperones, hormone transporters, or tumor suppressors [7-8]. They are involved in processes as diverse as dorsal-ventral axis formation, chromatin condensation in chicken erythrocytes and immunoregulation in Drosophila. For instance Maspin, a member of clade B has a RSL which is shorter than most RSL of the serpin superfamily and that is involved in cell migration [9]. Pigment Epithelial-Derived Factor (PEDF) is also a non inhibitory serpin with neuroprotective and antiangiogenic effects [8]. When nitrosylated, the alpha antitrypsin increases insulin-induced mitogenesis in various fibroblast and epithelial cell lines, and inhibits cell growth in human plasma [10-11].

Phylogenetic evolution

The evolutionary origin of serpins is unclear although their distribution suggests that serpins may have arisen early in eukaryotic evolution [12]. The clade B consists of 13 members in humans. Their genes are located at two genomic loci: 6p25 (SERPINB1, -6 and -9) and 18q21 (SERPINB2, -3, -4, -5, -7, -8, -10, -11, -12 and -13). They display seven or eight exons.

Two models exist to explain the presence of two clade B loci in humans. The first proposes duplication of the entire 6p25 locus followed by several single gene duplications at 18q21. The second proposes the opposite: the duplication of a single gene from 18q21 to 6p25, followed by successive duplications in this location [3].

SERPINB1

SERPINB1 (that has different names depending on the species: human MNEI; mouse elastase inhibitor, horse and pig Leukocyte elastase inhibitor) is an inhibitor of neutrophil elastase present particularly in granulocytes and macrophages, although it can be found at different concentrations in all tissues. SERPINB1 inhibits both elastase-like and chymotrypsin-like proteases through two distinct P1 (amino acid in the Nt direction of the cleaved bond) residues [13]. This serpin is in the group formerly called ov-serpin subfamily, which includes plasminogen activator inhibitor 2 and squamous cell carcinoma antigens 1 and 2 (SCCA1 and SCCA2) and maspin [3, 14].

SERPINB1 is an intracellular protein and acts primarily to protect the cell from proteases released into the cytoplasm during stress. This view is supported by KO mice which have a decreased viability of neutrophils [15]. This is consistent with its role as a protector of these cells from their own granular proteases. This function has been recently highlighted using myeloid conditional knock out and transgenic mice. In this paper Burgener et al clearly show the correlation between neutrophil survival and the level of SERPINB1 (B1a in mice) [16] .

Because of the lack of a cleavable hydrophobic signal sequence, SERPINB1 resides mainly in cytosol. It is also detectable in bronchoalveolar lavage fluid during lung inflammatory diseases. A lot of work has been performed investigating the impact of SERPINB1 on the control of extracellular neutrophil elastase released during chronic infections, particularly during respiratory diseases [17-18]. SERPINB1 has also been described as a potent inhibitor of human granzyme H as well as elastase, cathepsin G and D, and proteinase-3 [19]. Other chymotrypsin-like proteases, such as chymase, and chymotrypsin, were also reported to interact with SERPINB1 [13].

Studies using SERPINB1-deficient mice established the significant role for SERPINB1 in protecting lung antimicrobial proteins from proteolysis during microbe infection and its regulatory role in sustaining the balance of neutrophil reserve [15]. Neutrophil serine proteases (NSPs) (elastase, cathepsin G, and proteinase-3) kill invading microbes [18]. However, excess NSPs in the lungs play a central role in inflammatory pulmonary disease. On infection, wild-type (WT) and SERPINB1-KO mice show similar early responses, production of cytokines and chemokines, recruitment of neutrophils. However, SERPINB1$^{-/-}$ mice fail to efficiently clear bacteria due to a deficient recruitment of neutrophils to the lung because these cells show a shorter survival and increased release of neutrophil proteases in the extracellular space generating sustained pro-inflammatory cytokine production. The negative impact of overproduction of pro-inflammatory cytokines has been established by its association with fatal outcome of human influenza [15, 20]. The administration of a recombinant SERPINB1 protein to SERPIN KO mice normalized bacterial clearance, indicating that the regulation of pulmonary innate immunity by SERPINB1 is a non redundant key process during the host response to infection. In line with this, other results suggest that SERPINB1 may be a novel marker of active ulcerative colitis and may play an important role in the pathogenesis of inflammatory bowel disease, since it is seen accumulated in the intestinal lumen and in intestinal epithelial cells of patients [21]. Taken together these results indicate that SERPIN B1 has an important role in the resolution of inflammatory diseases.

LEI derived-DNase II: another function of SERPINB1

The eye lens is a useful model for the examination of many fundamental processes. Cellular differentiation of the lens is accompanied by nuclear degeneration morphologically related to nuclear modifications seen during apoptosis [22]. This includes the degradation of DNA in oligonucleosomes. We examined in detail this phenomenon and obtained results indicating that single strand and double strand cleavage of DNA cumulate in lens fiber nuclei [23]. However, the absence of 3'OH ends directed our attention towards the involvement of a DNase II (an endonuclease that releases 3' phosphate ends) in this process. By using polyclonal antibodies, done in our laboratory, directed against DNase II, purified from porcine spleen, we showed that the enzyme responsible for lens differentiating cell nuclei has a cytoplasmic localization in chick lens epithelial cells (that is not differentiated cells), but it concentrates in the nuclei of fibers cells [24]. Moreover, only this specific anti-DNase II antibody was able to inhibit the degradation of DNA from fibers cells *in vitro*. The crucial role played by DNase II in these processes drove our interest to its molecular structure, that was totally unknown by this time. We have then undertaken the cloning and molecular analysis of this protein. To do this, we first sequenced the protein by Edman degradation. Search for similarities of the obtained sequences within the databases showed 100% identity of our peptides with a protein of the serpin superfamily, the leukocyte elastase inhibitor (LEI), afterwards called SERPINB1 [25]. This was very intriguing: we sequenced an endonuclease of 27 kDa and obtained the sequence of an anti-protease of 42 kDa. Many experiments were necessary in order to prove that this was not a technical error.

Explaining these results was a major challenge. Many hypotheses were investigated [26]. Finally, the hypothesis of a post-translational modification was retained as explained below. Actually, after cloning the cDNA in bacteria we produced a protein of 42 kDa with an anti-protease activity, as expected. The protein, purified using its His-tag had not DNase activity. However, if we purified the recombinant protein not by using its His-tag but by applying the same protocol used to purify DNase II from pig spleen, we generated a 35 kDa protein that did have an endonuclease activity [25]. The same effect was obtained if we submitted the recombinant protein purified by the His-tag to an acidic pH, a mandatory step in the purification of DNase II from tissues. These results allowed us to conclude that the DNase activity was derived from LEI by post-translational modification. We therefore called it L-DNase II for LEI-derived DNase II. Later on, we showed that the acidic treatment could be replaced by elastase cleavage and that the elastase cleaved form of LEI had also a DNase II activity.

Molecular characterization of LEI/L-DNase II.

As mentioned above, treatment of LEI with elastase induces a decrease of its apparent molecular weight. This is linked to a loss of its anti-protease activity and to the appearance of an endonuclease activity. Hence, this protein has the fascinating capacity of changing its enzymatic activity. Several years later we collected enough arguments to support the idea that this intriguing property relays on the conformational modification seen in most serpins.

LEI, like most serpins, is a metastable protein and its anti-protease activity is related to its "stressed to relaxed transition". So that, the inhibition of elastase by the well described

suicide mechanism generates the cleaved form of LEI (see Padron-Barthe et al and Huntington et al [27] [6] for the detailed description of the mechanism). Interestingly, the analysis of the three-dimensional crystal structure of cleaved equine LEI showed a quite polarized molecule with a higher number of positive charges in the RCL pole. This is a necessary condition to interact with DNA, which is negatively charged. (fig 1).

Although we could not reveal any consensus sequence for endonuclease activity, analysis of LEI three-dimensional structure showed the presence of two histidines (H287 and H368) in the more positively charged region [27]. From these histidines, we focused on H368 as a candidate for being important at the active site because it is conserved in all species. The introduction of a point mutation on this residue (H368A) results in a molecule that conserves its anti-protease activity but has lost its endonuclease activity. These results suggest that the insertion of the RCL in the main beta-sheet uncovers a pre-existing endonuclease active site, related to H368. Moreover, our site-directed mutagenesis studies also showed that the RCL also hides a bipartite nuclear localization signal (NLS) which becomes exposed at the same time that the endonuclease active site, allowing then the nuclear translocation of the cleaved molecule. A molecular model of the shift in activity and cellular localization of LEI is seen on figure 2.

The containment of DNA in the cell nucleus permits the separation of the genomic information from processes located in the cytoplasm. This is important to regulate the activity of some proteins. The general means of controlling the proteins shuttle between nucleus and cytoplasm is through the presence of an NLS (allowing the protein to be introduced in the nucleus) and, in some proteins, of a Nuclear Extrusion Signal (NES). The NES consists on a motif containing three or four hydrophobic residues that bind a soluble export carrier: Crm1/exportin1. We have found a consensus NES sequence in LEI that binds Crm1 and ensures the nuclear export of the molecule [28].

Taken together these results allowed us to conclude that LEI/SERPINB1 intrinsically possesses two enzymatic activities: an anti-protease activity dependent on its reactive site loop, which is analogous to the other proteins of the family and an endonuclease activity which is unveiled by the cleavage of the RSL by the cognate protease after the conformational modification induced by this cleavage, thought the same mechanism of inhibition of all serpins [6]. In addition the conformational change also unveils a bipartite NLS allowing the protein to translocate to the nucleus. LEI displays also a functional NES sequence allowing to control, by nuclear exclusion of the DNase activity of L-DNase II.

LEI/L-DNase II in apoptosis

The orderly cell dismantling seen in apoptosis involves proteolysis and chromatin degradation. The degradation of DNA in oligonucleosomes (DNA laddering) was considered in the earliest 90' as the molecular hallmark of apoptosis. The search of DNases became then an important goal in apoptosis research [29]. Many enzymes were proposed as candidates, but most did not fulfill the criteria for the apoptotic DNase. At that time, many results indicated the existence of different apoptotic pathways but these results were neglected due to the increasing knowledge on caspases activation and the discover of CAD (Caspase-Activated DNase). Since then it has been shown that the apoptotic phenotype of a dying cell can be obtained by the activation of other proteases, including calpains, cathepsins

and granzymes [30]. These proteases may activate, directly or indirectly, other endonucleases than CAD. To date, only the activation of: GAAD (Granzyme A-Activated DNase) and L-DNase II have been related to the activation of proteases [31-32]. Of note, the release of AIF (Apoptosis Inducing Factor) has also been related to protease activities (calpains) although its action on DNA is not to cleave it but to condense chromatin [33].

L-DNase II: a pro-apoptotic molecule

As described previously the route to L-DNase II discovery was open by the nucleus degradation during lens cell differentiation in the chick. Interestingly, the morphological changes of the differentiated nuclei are similar to those found in the nuclei of apoptotic cells [22]. Thus, we investigated the involvement of L-DNase II in apoptosis. To do this we purified nuclei form several cell lineages. The incubation of these nuclei with recombinant L-DNase II induces the cleavage of DNA into an oligonucleosomal ladder *in vitro*, inducing, also, nuclear pycnosis [25]. In cellular models a nuclear translocation of the enzyme is seen during apoptosis when using inductors leading to a metabolic stress [34]. In these paradigms the overexpression of LEI enhances cell death. It is important to note that genotoxic compounds do not seem to activate L-DNase II. In this case the overexpression of LEI does not increase cell death, underling the role of LEI cleavage and the generation of L-DNase II in triggering specific forms of cell death.

The activation of L-DNase II was seen in neural apoptosis during retina development [35] or in cell culture in different cell lines [34, 36-39], as well as in pathological conditions like corneal graft rejection and light induced retinal degeneration (LIRD). In most cases the activation of this pathway seems to be dependent on metabolic stresses but not on genotoxic injuries, as said before. L-DNase II is mostly activated in differentiated cells, or in tumor cells unable to activate the caspases pathway [40]. Different proteases can transform LEI into L-DNase II: intracellular elastase, a neutral protease and cathepsin D, an acid protease [37, 41-42]. Other elastase-like proteases activated during apoptosis-like cell death were also shown to be able to activate L-DNase II. This is the case of AP-24 (apoptotic protease 24 kDa) or other serine proteases [36, 43].

It is worth to note that as a primary response to infection neutrophils expel their DNA to form neutrophil extracellular traps (NETs) to ensnare bacteria before dying. NETs formation involves the degradation of the nuclear envelope and DNA release into the cell, culminating with neutrophil lysis. The NETs contain histones and elastases. The presence of LEI should be interesting to investigate, owing to its capacity of controlling elastase activity and DNA cleavage [44].

LEI: an anti-apoptotic molecule

Many members of the ov-serpins family are considered cytoprotective. Actually, Most of the serpins of clade B regulate enzymes involved in cell death. Some regulate lysosomal proteases (squamous cell carcinoma antigen), some can inhibit caspases. This is the case of the viral serpin Crm A that inhibits Fas or TNFa-induced apoptosis [45]. Likewise, overexpression of PI-9 protects cells from granzyme B induced apoptosis [46] . As many members of the clade B reside in protease-secreting cells, it was suggested that they protect the cells from auto-destruction by misdirected proteases, as said above. In addition,

exogenous ov-serpins may protect neighboring cells and tissues from proteolytic damage. In line with this, recombinant LEI delivered to the airways of rats, for instance, prevents lung injury by neutrophil proteases. This point to the interest of this protein in treating inflammatory lung diseases [18]. According to these data it is not surprising that LEI in its native form has also anti-apoptotic properties.

We investigated the basis of the anti-apoptotic activity of LEI in etoposide-induced apoptosis and show that it is indirectly mediated through the inhibition of cathepsin D. Following etoposide treatment, cathepsin D is released into the cytoplasm by lysosomal membrane permeabilisation (LMP) and cleaves procaspase-8 inducing a burst of caspase-8 activity [42]. This pathway can be slowed down by LEI overexpression. An important feature is that the AP10T-LEI mutant, bearing a mutation that slows down the insertion of the reactive site loop in the main beta sheet, leading to a decrease in the stability of the covalent complex between cathepsin D and LEI, abolish the cytoprotective activity of the protein. This led us to the conclusion that the anti-protease activity of LEI is essential for its binding to cathepsin D and that LEI probably inhibits cathepsin D in a serpin-like manner, although the point of cleavage on LEI has not been established [42].

It is interesting to note that LEI is an ubiquitous protein (figure 3). However, its level of expression is very variable in different cells and tissues. In this way, tissues expressing high levels of LEI may use this protein to control caspase activity together with others previously described caspase inhibitors like IAPs (Inhibitors of Apoptosis Protein).

Neutrophils are the first responders of the inflammatory response. They are characterized by their potent cytotoxic content but also by their limited lifetime. This short half-life is thought to be a protecting mechanism for the host, as underlined by the numerous pathologies associated with imbalanced neutrophil survival. Neutrophil death is the prototype of programmed cell death, dependent on the activation of caspase-3. In aging neutrophils, the activation of caspase-3 is mediated by Proteinase 3 (PR3), independently of the canonical extrinsic and intrinsic apoptosis pathways. PR3 is normally stored in granules and is progressively released to the cytosol during neutrophil aging. Once in the cytosol, PR3 cleaves procaspase-3. SERPINB1 counterbalances the activity of PR3 and its deletion in neutrophils accelerates their spontaneous death. By this means SERPINB1 can also control classical apoptosis [19].

Protein-protein interactions of LEI/L-DNase II and the control of cell death

It is likely that the pro- or anti-apoptotic effects of LEI/L-DNase II depend both on the molecular transformation of LEI into L-DNase II but also on the interaction of these molecules with other pro-apoptotic or pro-survival pathways and their regulating molecules. A screening of protein–protein interactions between LEI and cellular proteins involved in apoptosis was performed in our laboratory revealing several candidates to LEI/L-DNase II regulation. Some of them have been carefully analyzed as described below.

PARP-1

The most widely described substrate of caspases is poly(ADP-ribose) polymerase-1 (PARP-1 – EC 2.4.2.30), an enzyme that detects DNA breaks and uses NAD^+ to form poly(ADP-ribose) (PAR) polymers and bind to various protein acceptors, assisting DNA repair [47]. *In silico* studies showed that LEI/L-DNase II contains a PAR-binding domain, which could promote its interaction with PARP-1. We investigated then the possible interaction between PARP-1 and LEI [48-49].

After showing that LEI/L-DNase II and PARP-1 co-immunoprecipitate from HeLa cells extracts, we found that neither LEI nor L-DNase II are acceptors of PAR. In line with this, their respective anti-elastase and endonuclease activities are not affected by PARP-1 interaction, demonstrating the absence of a regulation of this DNase by poly (ADP-ribosylation). This also suggested that the putative PAR-acceptor sites identified *in silico* were not efficient *in vitro*. However, the LEI/PARP-1 interaction induces an increase of the auto-poly (ADP-ribosylation) of PARP-1, a post-translational modification that down regulates PARP activity. This effect is enforced by the overexpression of LEI.

It is worth to note that during caspase-dependent apoptosis, early and transient stimulation of PARP-1 causes PAR accumulation in apoptotic cells [50]. To prevent excessive NAD and ATP depletion, caspase-3 cleaves PARP-1, thus producing fragments, lacking enzymatic activity. Also, PARP-1 is able to negatively regulate CAD. As a result, inactivation of PARP-1 by caspases is required for apoptotic DNase action [51]. Our data suggest that the physical association between PARP-1 and LEI has a different functional role. We observed not only that LEI activity is not modulated by poly (ADP-ribosylation) but also that LEI can stimulate PARP-1 activity. In the apoptotic context, the increase of the auto-ADP ribosylation of PARP-1 (which is inhibitory) could represent a way to block the activity of this enzyme when caspases are not activated.

BCL2 family of proteins

The mitochondrial pathway of caspases-dependent apoptosis is tightly regulated by the BCL-2 (B-cell lymphoma 2) family of proteins, which is composed by 3 groups of members: the anti- and pro-apoptotic members that control mitochondrial membrane permeabilisation (MMP) and the BH3-only proteins that detect the apoptotic signal and activate pro-apoptotic proteins. For instance, BAX activation allows its homo-oligomerization and pore formation in the mitochondrial membrane through which pro-apoptotic factors will be released [52]. This process is antagonized by the anti-apoptotic members of the BCL-2 family through a complex interplay of protein-protein interactions. The pores formed allow the release of cytochrome C, that triggers apoptosome formation of other caspase-independent apoptotic factors like AIF (Apoptosis Inducing Factor), HrtA/Omi or endonuclease G [53-54]. In spite of this, MMP is not involved in all apoptotic cell deaths. LEI/L-DNase II uses cytoplasmic effectors (serine and lysosomal proteases) and does not require MMP. Though, the interaction of this molecule with proteins regulating MMP does not seem necessary *per se*.

Our interactome study, however, showed a possible interaction with this family of proteins. Therefore, two members of this family BCL-2 (belonging of the anti-apoptotic group) and BAX (belonging to the anti-apoptotic group) were analyzed.

By performing pull down, co-immunoprecipitation and glycerol gradient experiments we showed that LEI/L-DNase II interacts with BCL-2 family members in apoptotic and non-apoptotic conditions [55]. The interaction BCL-2/LEI/L-DNase II is protective, confirming the already known anti-apoptotic function of BCL-2. However, the interaction with the classical pro-apoptotic protein BAX does not have the expected effect and turns out to be also protective in L-DNase II mediated apoptosis. This result is surprising at the first glance but, in fact, it highlights the importance of the intracellular environment of the protein to the determinism of its function. Actually, in L-DNase II mediated apoptosis, BAX acquires a nuclear location, a feature that raises the possibility that BAX may lose its classical pro-apoptotic function when sequestered to the nucleus. The analysis of LEI and the BCL-2 family proteins by local alignment of multiple alignment (LAMA) and multiple sequence alignment motifs (blocks) suggests the existence of an interacting alpha helix in the region 281-311 and a "canonical" interacting sequence in the helix spamming the region 231-254 of LEI [55]. However, an interaction study using purified molecules in which these helixes are destabilized by site-directed mutagenesis is lacking.

Protein kinase C zeta

Atypical protein kinase C (PKC) zeta is a serine-threonine kinase involved in cell protection against various stresses [56]. Our laboratory has shown that this kinase is activated in the retina following intravitreal administration of tumor necrosis factor (TNF) [57] and that is cytoprotective in endotoxin-induced uveitis (EIU) [58]. As many PKCs, it is a cytoplasmic protein which is translocated to the plasma membrane or to the nucleus upon activation. It has been shown that following stress, activated PKC zeta phosphorylates Inhibitor kappa kinase B (IkB), which leads, to the release of Nuclear Factor Kappa B (NFkB) and its nuclear translocation. NFkB promotes the transcription of anti-apoptotic genes such as Bcl-2, inhibitor of apoptosis protein, TNF receptor-associated factors [59]. So that, activation of PKC zeta and NFkB pathways acts as inhibitory mechanisms of cell death in stress response. We had previously shown that in LIRD photoreceptor cell death is mediated by L-DNase II and that PKC zeta is activated [60]. Pull-down and co-immunoprecipitation studies indicate that both molecules can interact. *In silico* studies identify a consensus phosphorylation site for LEI/L-DNase II and the site directed mutagenesis of this site, mimicking a phosphorylation, impairs L-DNase II nuclear translocation. More importantly, when PKC zeta is inhibited by siRNA in a cell model of LEI activation, L-DNase II increases in the nucleus and the survival of these cells is decreased. In line with this, the L-DNase II activity is increased in light exposed retinas if PKC zeta has been inhibited by the intravitreal injection of its inhibitor [60].

Taken together these results indicate that the nuclear translocation of L-DNase II, a key feature in its pro-apoptotic activity can be regulated by a pro-survival kinase, PKC zeta.

Apoptosis Inducing Factor

Apoptosis Inducing Factor (AIF) is a highly conserved flavoprotein located in the intermembrane space of mitochondria, where it maintains and organizes the respiratory complex I [61]. This function was confirmed in a conditional KO mice that exhibits impaired

respiratory chain activity and develops severe cardiomyopathy and skeletal muscle atrophy [62]. Moreover, the Harlequin mouse in which only 20 % of AIF is expressed, cerebellar granule cells are more susceptible to cell death than wild-type littermates [63]. Upon an apoptotic stimulus AIF is cleaved by calpain I and translocated to the nucleus where it triggers chromatin condensation [33]. Enzymatic studies show that AIF does not display endonuclease activity and DNA-degrading activity depends on the recruitment of nucleases [64]. We have shown that AIF and LEI/L-DNase II interact and that they can cooperate. *In vitro*, using purified nuclei, we showed that the chromatin condensation properties of AIF are enhanced in the presence of L-DNase II and the endonuclease activity of L-DNase II is increased in the presence of AIF. *In vivo*, the overexpression of AIF and LEI enhances the pro-apoptotic activity of each molecule. On the contrary, suppression of their expression protects cells. Moreover, pull-down and co-immunoprecipitation experiments show that these molecules can interact and are translocated to the nucleus during cell death [65]. Interestingly, this pattern of action, in which a DNA condensing molecule and an endonuclease collaborate in nucleus dismantling, is also seen in classical apoptosis. In caspase-dependent apoptosis nuclear degradation involves the activation of CAD that degrades DNA but also of a molecule condensing the chromatin: acinus [66]. Both are cleaved by effectors caspases, mostly caspase 3, and translocated to the nucleus where they induce DNA condensation and cleavage. AIF has been identified as a very important player in caspase-independent cell death and it has been shown to interact with many proteins such as PARP-1, calpains, Bax, Bcl-2, histone H2AX [67].

According to our observations, L-DNase II joins cyclophilin A and endonuclease G on the list of enzymes that are reported to be activated by AIF. In the case of L-DNase II the interaction with AIF increases the pro-apoptotic effects of both molecules. Importantly, these interactions are not mandatory and strongly depend on the apoptotic stimulus. For instance, the induction of apoptosis by Hexamethylene amiloride activates L-DNase II but not AIF.

As we stated before, LEI/L-DNase II is able to activate PARP-1. Actually, the overexpression of LEI induces a sustained production of PAR [48]. Interestingly, the accumulation of PAR in the cell has been proved to be important for the mitochondrial release of AIF [68]. In long cultured HeLa cells, where both AIF and L-DNase II are activated, we have shown that L-DNase II precedes AIF in the nucleus. This allows us to hypothesize that nuclear L-DNase II may contribute to the accumulation of PAR and thus to the nuclear translocation of AIF. This does not mean that L-DNase II activation is a mandatory step in AIF release. In models where L-DNase II is not activated (apoptosis induction by MNNG, for instance) AIF is still nuclear translocated owing to the activation of PARP-1 through DNA damage. In the same way, the presence of AIF is not necessary to produce LEI cleavage as we have shown by the use of AIF KO cells [65]. It seems then, that we have here two pro-apoptotic systems that can work by themselves but that can also cooperate in order to execute efficient cell demise. It is also worth noting that AIF and L-DNase II are both effectors of caspase-independent cell death that link the activation of non caspases proteases, e.g. calpains, cathepsins and serine proteases to DNA degradation in the same way that caspase activation is related to CAD.

Evolutionary tips

The serpin inhibitory mechanism is extremely well adapted to evolutionary changes because a single amino acid substitution in the RCL can led to the inhibition of a totally

different protease. We have just seen the surprising capacity of SERPINB1 to become an endonuclease but other serpins have developed analogous properties, related to DNA status. For example, a1-antichymotrypsin (inhibitor of cathepsin G and chymase), is able to inhibit purified DNA polymerase and DNA primase [69]. MENT (Myeloid and Erythroid Nuclear Termination stage-specific protein), is an inhibitor of cathepsins K, L, and V, as well as a cysteine proteinase from CV-1 cells [70]. It is a nuclear protein present in the avian blood cells where it is the predominant non-histone protein. MENT imposes a dramatic remodeling of chromatin by forming "bridges" between nucleosomes, bringing about a strong repressive effect on cell proliferation.

Other functions of SERPINB1

In the paragraphs above we have seen that SERPINB1 is able to inhibit several proteases and to degrade DNA when cleaved. Due to these two properties, SERPINB1 is able to control cell survival having, dependent on the cell condition, anti or pro-apoptotic properties. Recent results from our and others laboratories indicate that the diversity of functions of this protein are not completely elucidated.

On one hand, we have recently shown that in bovine corneal epithelium LEI increases its expression early after injury and returns to basal levels immediately after wound closure [71]. This increase is blocked by N-acetylcysteine, suggesting that production of reactive oxygen species immediately after wounding is involved in this overexpression.

On the other hand, SERPINB1 expression was shown to be down-regulated in high-grade human glioma and glioblastoma cell lines. Overexpression of SERPINB1 suppressed, while knock-down of SERPINB1 promoted, the migration and invasion of glioma cells. Further studies conducted on glioma cells indicate that SERPINB1 inhibited glioma migration and invasion by dampening the expression of matrix metalloproteinase-2 (MMP-2) and by inactivating focal adhesion kinase (FAK) phosphorylation, which is involved in the down regulation of MMP-2 [72]. Moreover, in human keratinocyte cell line (HaCaT) proliferation was decreased by treatment with calcitriol which is mediated by an up regulation of SERPINB1 [73]. Other studies suggested that SERPINB1 may suppress the migration and invasion of lung and breast cancers as well as hepatocellular carcinoma cells.

However, the differential proteomes of two oral cancer cells, CAL-27 and SAS, with the highest and the lowest migration potential, respectively, show that SERPINB1 was highly expressed in CAL-27 [74]. In this case expression of SERPINB1 correlated positively with cell migration. This was supported by the ectopic expression of SERPINB1 in oral cancer cells, in which the overexpression of the protein increased cell migration.

Taken together these results suggest that SERPINB1 may be involved in cell migration, while its final effect on this parameter depends on the type of cell.

Conclusion

SERPINB1, LEI/L-DNase II is a protein with at least two enzymatic activities and an important role in the regulation of cell survival. Its role in inflammation is clear due to its involvement in the resolution of chronic inflammatory lung and bowel diseases. Recent data

indicate that it has also a role in cell migration suggesting that it could be involved in diverse processes like wound healing and malignant metastases. The existence of rodent-specific paralogues in several clades (especially in clade B) is consistent with a larger number of proteases in these species but complicates the use of these animals as experimental KO models because complementary or compensatory effects are difficult to exclude.

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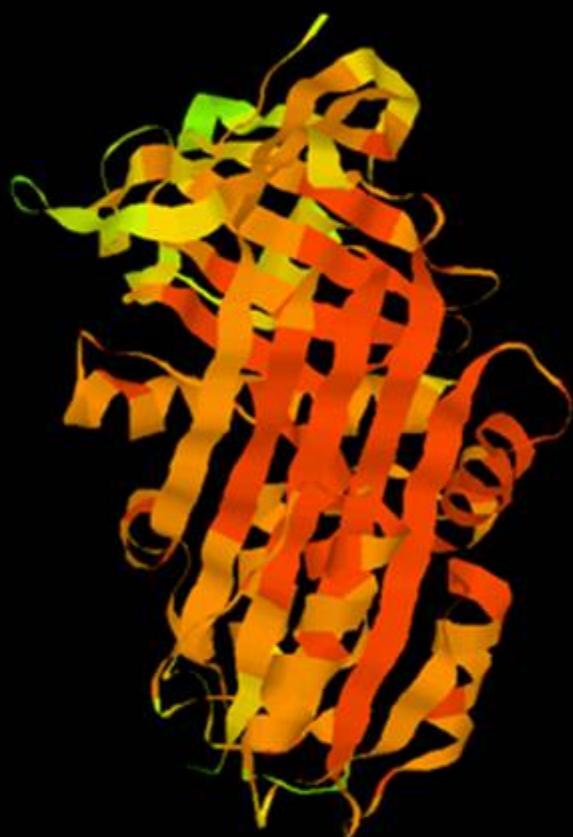
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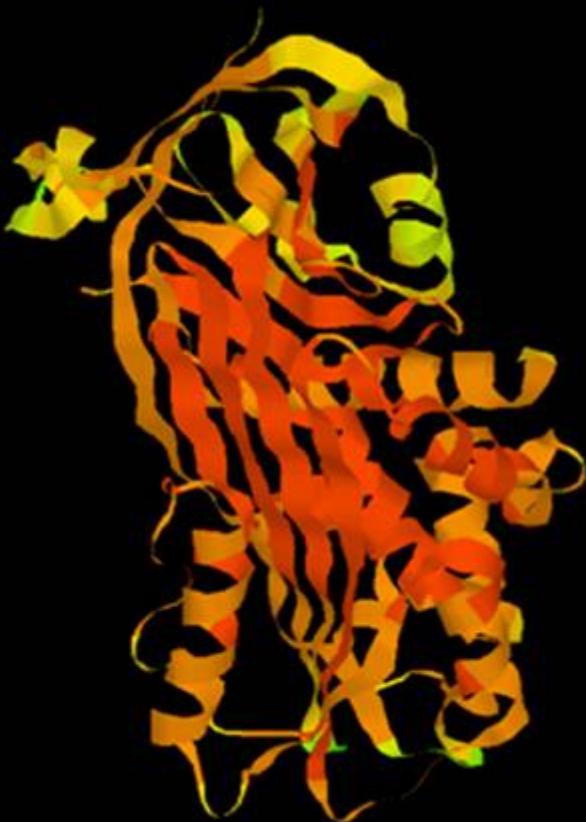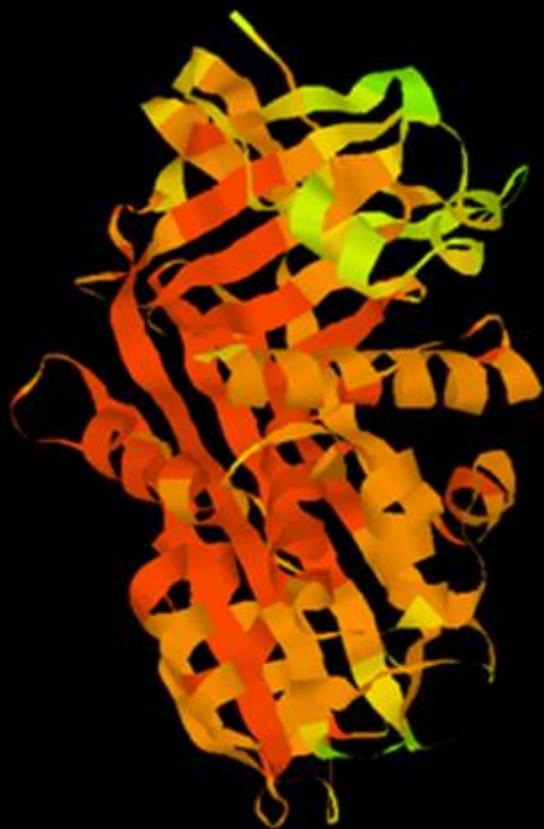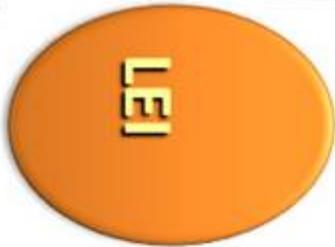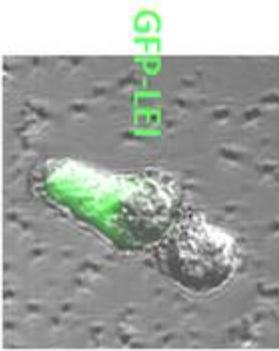
Leukocyte
Elastase Inhibitor

Cytoplasmic
Anti-protease
Anti-apoptotic

Inactive
Protease

LEI

Apoptotic signal

cleavage

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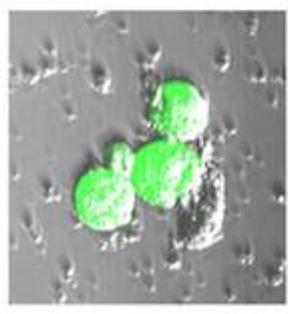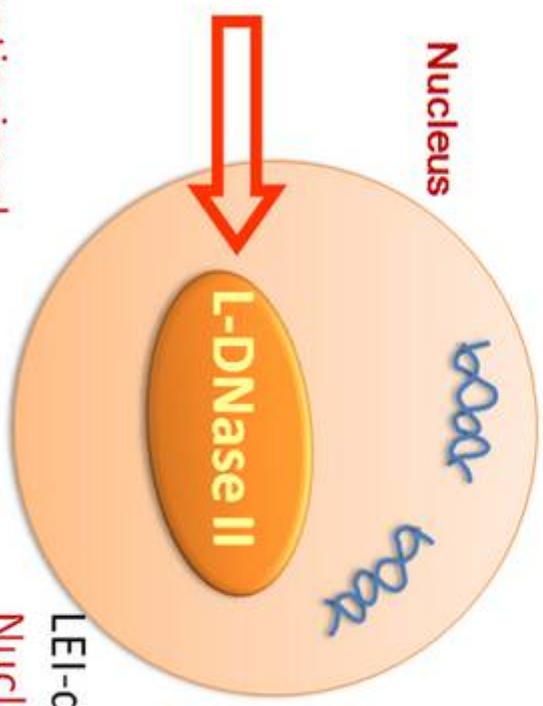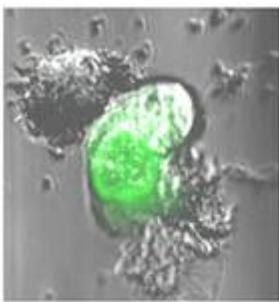
L-DNase II

LEI-derived DNase II
Nuclear
Endonuclease
Pro-apoptotic

Active
Protease

GFP-LEI

Pig

Liver Intestine Pancreas Lung Spleen Kidney Ovary

Rat
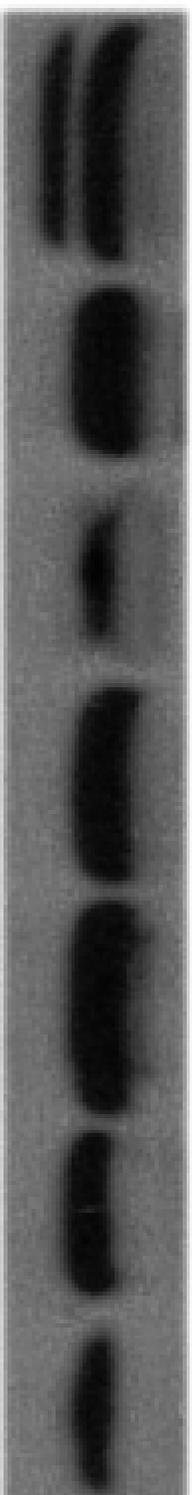
Brain Retina Lens Intestine Spleen Thymus Blood Ovary LEI